Novel application and serial evaluation of tissue-engineered portal vein grafts in a murine model

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Aim: Surgical management of pediatric extrahepatic portal vein obstruction requires meso-Rex bypass using autologous or synthetic grafts. Tissue-engineered vascular grafts (TEVGs) provide an alternative, but no validated animal models using portal TEVGs exist. Herein, we preclinically assess TEVGs as portal vein bypass grafts. **Materials & methods:** TEVGs were implanted as portal vein interposition conduits in SCID-beige mice, monitored by ultrasound and micro-computed tomography, and histologically assessed postmortem at 12 months. **Results:** TEVGs remained patent for 12 months. Histologic analysis demonstrated formation of neovessels that resembled native portal veins, with similar content of smooth muscle cells, collagen type III and elastin. **Conclusion:** TEVGs are feasible portal vein conduits in a murine model. Further preclinical evaluation of TEVGs may facilitate pediatric clinical translation.

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Children diagnosed with extrahepatic portal vein obstruction (EHPVO) are at increased risk of gastrointestinal bleeding, protein-losing enteropathy, malabsorption caused by chronic intestinal congestion and symptoms of severe hypersplenism [1]. The etiology of EHPVO includes perinatal events such as umbilical catheterization, sepsis and prothrombotic disorders [2]. Patients initially possess normal intrinsic hepatic function. In the face of recurrent severe bleeding or significant hypersplenism, portosystemic shunts have been widely used to redirect portal blood flow; however, these shunts fail to restore physiologic hepatic portal perfusion and encephalopathy remains a persistent risk [1].

The approach to the management of portal hypertension in children has evolved significantly over recent decades. Since its description by de Ville de Goyet in 1992 [3], mesenterico-left portal vein (meso-Rex) bypass has been widely adopted as an efficacious intervention for EHPVO. Connection of the superior mesenteric vein to the left portal vein alleviates the risk of severe gastrointestinal bleeding and other sequelae of sustained portal hypertension while restoring physiologic blood supply to the liver [2,4,5].

For neonates or small children, the meso-Rex technique is a favored approach due to preservation of somatic growth potential [6]. Autologous internal jugular veins are the most common vascular conduits used for this purpose. In cases where the jugular vein is not accessible or otherwise unusable, recanalized umbilical veins have also been described as a feasible alternative [7]. These options are preferable to synthetic grafts, which possess an inherent risk of infection and thrombosis while also lacking growth potential [8]. However, harvesting of veins is associated with risks to the patient and surgeons are commonly confronted with the obstacle of inadequate vein caliber or length.

Due to off-the-shelf availability, capacity for growth and avoiding the need for harvesting a venous conduit, tissue-engineered vascular grafts (TEVGs) may offer additional benefits over traditional autologous or synthetic conduits

Figure 1. Surgical Implantation of tissue-engineered portal vein in a murine model. (A) Scanning electron microscopy of biodegradable scaffold, with porous microstructure allowing for cellular infiltration. (B) Intraoperative photos demonstrate native murine portal anatomy (left), TEPV during surgical implantation (middle) and after surgical implantation (right). No significant changes in (C) body weight or (D) spleen weight were observed between mice implanted with TEPVs and age-matched controls.

IVC: Inferior vena cava; mos: Months; PV: Portal vein; TEPV: Tissue-engineered portal vein.

as meso-Rex bypass vascular grafts [8]. Our research team has preclinically validated and clinically translated TEVGs for palliation of congenital heart disease for children undergoing modified Fontan procedures [9–13]. Importantly, requirements of a TEVG intended for extracardiac total cavopulmonary connection are similar to those for a meso-Rex bypass, where both systems are characterized by high flow and low hemodynamic pressure, along with similarities in vessel caliber and length. Therefore, in the present study, we evaluated the feasibility of implanting TEVGs as portal vein interposition grafts in a murine model and serially assessed the remodeling characteristics of TEVGs using *in vivo* imaging and *ex vivo* analysis.

Materials & methods

Vascular graft scaffolds

Scaffolds were constructed using a dual cylinder chamber molding system from a nonwoven polyglycolic acid fiber mesh (Concordia Fibers, RI, USA) and a 50:50 poly (ϵ -caprolactone-co-L-lactic acid) copolymer (PCLA) sealant (Gunze Co. Ltd., Kyoto, Japan), as previously described [14]. Each scaffold was 3 mm in length and 0.8 mm in diameter (Figure 1A).

Vascular graft implantation

Grafts were surgically implanted in 10-week-old female SCID-beige(bg) mice ($n = 12$), which our research team had previously utilized for TEVG implantation and had demonstrated good rates of patency and low rates of thrombosis [15–18]. Mice were anesthetized using ketamine 100 mg/kg (Hospira Inc., IL, USA) and xylazine

10 mg/kg (Lloyd Inc., IA, USA) cocktail with ketoprofen 5 mg/kg (Fort Dodge Animal Health, IA, USA) as pre-anesthesia analgesic. The surgical site was shaved and then disinfected with betadine and alcohol pads. A midline laparotomy incision from below the xyphoid to the suprapubic region was made, and a self-retaining retractor inserted. The intestines were wrapped in saline-moistened gauze and retracted. Surgery was performed under microscopic guidance. Specifically, the main trunk of the portal vein was exposed and dissected. Vascular control was obtained with microvascular clamps and the portal vein was transected. A portal vein interposition graft was implanted with proximal and distal end-to-end anastomoses using sterile 10-0 monofilament sutures on tapered needles. Any hemorrhage was controlled with Surgicel, a topical absorbable sterile hemostatic agent (Ethicon, NJ, USA). The intestines were returned to the abdominal cavity, and the abdominal musculature and skin were closed in two layers using 6-0 prolene suture (Ethicon US, LLC, OH, USA; Figure 1B). A total of 12 grafts were successfully implanted in 12 animals (i.e., survival up to >24 h after operation). No antiplatelet or anticoagulant agents were used postoperatively. Tissue-engineered portal veins (TEPVs) were explanted at predetermined time points of 1 (n = 1), 2 (n = 1), 6 (n = 5) and 12 months (n = 5) postimplantation for analysis. Body and spleen weights were assessed for each animal at the time of sacrifice. All animals received humane care in compliance with the NIH Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee at Yale University approved the use of animals and all procedures described in this study.

In vivo ultrasound

Serial ultrasonography (Vevo VisualSonics 770; VisualSonics, ON, Canada) was used to monitor randomly selected TEPVs at 1 (n = 5), 3 (n = 6), 6 (n = 7), 10 (n = 5) and 12 (n = 2) months after implantation. Prior to ultrasonography, mice were anesthetized with 1.5% inhaled isoflurane. Graft luminal diameter was determined sonographically and patency was determined by measuring flow velocity proximal and distal to the graft.

In vivo micro-computed tomography angiography

Under anesthesia, *in vivo* micro-computed tomography (CT) angiography (GE eXplore Locus, GE Healthcare, WI, USA) was performed in a subset of randomly selected animals. 1 min prior to image acquisition, animals were administered an intrajugular 0.3-cc bolus of Ultravist (370 mgI/ml, Bayer Healthcare, NJ, USA). Micro-CT data were acquired with an x-ray source of 70 kVp tube voltage, 32 mA tube current, 4×4 detector binning model, 16 ms exposure per frame, 70 gain and 20 offset for contrast-enhanced CT acquisitions. A single frame of 220 projections for 42 s of continuous x-ray exposure was used. Volumetric micro-CT images were reconstructed in a $360 \times 180 \times 445$ format with voxel dimensions of $98.4 \times 98.4 \times 98.4 \mu\text{m}^3$ using a Feldkamp algorithm with calibrated Hounsfield units. Micro-CT data was transferred to an image processing workstation (Advanced Workstation, version 4.4, GE Healthcare) for further reconstruction and quantitative analysis. Portal grafts and anastomosis sites were identified in CT images. Measurements of inner luminal diameter and volume were assessed for TEPVs at 2 (n = 1), 6 (n = 1) and 12 (n = 2) months postimplantation, as well as in native portal veins from age-matched control SCID-bg mice (n = 5).

Histology

After explantation, graft tissue was fixed in 4% paraformaldehyde and embedded in paraffin. 5 μm -thick sections were then stained using standardized techniques for Hematoxylin & eosin, Masson's Trichrome (collagen) and elastica van Gieson (elastin).

RNA isolation & reverse transcription-PCR

Explanted TEPVs and native portal vein tissue were frozen in optimal cutting temperature compound (Tissue-Tek; Sakura Finetek, CA, USA), and sectioned into 20–30 μm sections using Leica CM 1950 cryostat (Leica Biosystems, Wetzlar, Germany) at 6 and 12 months after implantation. Excess optimal cutting temperature compound was removed by centrifugation in phosphate-buffered saline (PBS). RNA was extracted and purified using the RNeasy mini kit (Qiagen, Venlo, The Netherlands). Reverse transcription (RT)-PCR was performed using predeveloped assay reagents (Applied Biosystems, CA, USA), as previously described [19]. Primers for the following genes were used: smooth muscle cell (*vim*; Mm01333430.m1, Life Technologies, CA, USA), elastin (*eln*; Mm00514670.m1, Life Technologies), collagen type I (*col1a1*; Mm00801666.g1, Life Technologies) and collagen type III (*col3a1*; Mm01254476.m1, Life Technologies). HPRT1 (*Hprt*; Mm00446968.m1, Life Technologies) was used as a housekeeping gene.

Endothelial staining

Explanted TEPVs were cut longitudinally and fixed with stainless steel insect pins on a silicon block. Tissue was then fixed with 4% paraformaldehyde/PBS at 4°C for 30 min, after which the tissues were washed in PBS. The tissue was then incubated in 0.3%–TX100/2% BSA/PBS solution at room temperature for 15 min to achieve permeabilization. Next, the tissue was incubated with primary antibodies, including VE-cadherin (1:100, Santa Cruz Biotechnology, Inc., TX, USA) and eNOS (1:10, Novus Biologicals, LLC, CO, USA) overnight at 4°C. The following day, tissue was washed with PBS and incubated with secondary antibody (1:500) for 3 h. Finally, the tissue was washed with PBS, mounted with medium containing 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, CA, USA) and evaluated using a fluorescent microscope (Eclipse E800; Nikon Inc., NY, USA).

Statistical analysis

Differences in body and splenic weight were assessed by analysis of variance with Tukey correction for multiple comparisons. In the assessment of luminal diameter via ultrasound, group differences were analyzed using the nonparametric Mann–Whitney test for paired data. Analysis of variance with Tukey correction for multiple comparisons was utilized for analysis of group differences in the histological analysis of smooth muscle cell, elastin, collagen type I and collagen type III content. All statistical analyses were performed using commercially available software (GraphPad Prism v6.0 for Mac OS X, GraphPad Software, CA, USA). Statistical significance for all analyses was set at $p < 0.05$. All values are expressed as means $\pm$ standard deviation unless stated otherwise.

Results

Microsurgical implantation of TEPV as interposition portal vein conduits

12 TEPV implantations were successfully performed (Figure 1B). Operation time was approximately 45 min (clamp time: 20–25 min). The presence of portal hypertension was assessed by evaluation of splenic weight. There were no differences in body weight (Figure 1C) or splenic weight (Figure 1D) among mice at 6 and 12 months after implantation.

Serial ultrasound imaging

Implanted TEPVs were serially monitored by ultrasound to assess changes in both graft patency and diameter for up to 12 months (Figure 2). Average luminal diameter was 0.66 ± 0.16 mm at 1 month, 0.71 ± 0.11 mm at 3 months, 0.67 ± 0.05 mm at 6 months, 0.75 ± 0.08 at 10 months and 0.78 ± 0.05 at 12 months. Native portal veins (PVs) were also monitored by ultrasound and demonstrated an average luminal diameter of 0.78 ± 0.07 mm during the 12-month experimental time course.

Assessment of TEPV morphology using micro-CT angiography

In vivo micro-CT angiography was performed in a subset of animals at 2, 6 and 12 months after TEPV implantation. Age-matched control PVs were also analyzed at each time point (Figure 3). Due to difficulty in identifying sites of anastomosis, the length of the TEPV was approximated using a standard graft length of 3 mm. Analysis of luminal volume measurements revealed patent TEPVs with similar morphology to that of native veins (native PV: 4.74 ± 0.68 mm³; TEPV: 5.17 mm³ at 2 months, 4.62 mm³ at 6 months and 4.86 ± 0.25 mm³ at 12 months).

Histological & RT-PCR analysis

Cellular infiltration of TEPVs was seen as early as 1 month after implantation (Figure 4A). Nearly all scaffold fibers had been degraded by 6 months after implantation (Figure 4A). Qualitative histologic assessment of TEPV tissue explanted at 12 months after implantation demonstrated a TEPV wall that resembled native tissue, with the presence of three distinct layers including an intima containing endothelial cells, media containing smooth muscle cells and an adventitia (Figure 4A).

RT-PCR demonstrated increased smooth muscle cell content in TEPVs compared with native PVs at 6 months, but not 12 months after implantation (Figure 4B). Subjective analysis of extracellular matrix stains revealed deposition of collagen throughout TEPVs (Figure 4A). Further quantitative analysis confirmed increased expression of collagen type I at 6 and 12 months after TEPV implantation; however, no differences in collagen type III were found between TEPVs and native PV tissue at 6 or 12 months (Figure 4B). Elastin formation in TEPVs was significantly lower than native PV tissue at 6 months, but no significant differences in elastin content existed at 12 months after TEPV implantation (Figure 4B).

Figure 2. Serial assessment of *in vivo* ultrasound. (A) *In vivo* ultrasound images demonstrate TEPV patency at 2 weeks, 1, 6, 8 and 12 months after implantation in a representative animal. (B) Quantitative analysis of serial ultrasound imaging in TEPVs demonstrated a trend toward luminal narrowing at early time points followed by progressive dilatation at 12 months, at which time the TEPVs luminal diameter closely resembled that of native portal veins.
mos: Months; PV: Portal vein; TEPV: Tissue-engineered portal vein.

Immunohistochemical staining of TEPV & native vein endothelium

Endothelial staining in native PV and TEPV tissue demonstrated expression of VE-cadherin, a marker of the endothelial cell-to-cell junctions, along with the presence of eNOS and nuclei (DAPI), thus representing a trend where TEPVs appeared to transition into functional neovessels within 12 months after implantation (Figure 5).

Discussion

The clinical feasibility of a TEVG as a meso-Rex bypass graft was first described in a 2012 case report involving a 10-year-old girl [20]. In 2014, the same team of researchers demonstrated the clinical translation of TEVGs using decellularized allogeneic vascular scaffolds in two children requiring meso-Rex procedures [21]. However, little progress has occurred in this field aside from initial pilot studies, and scarce information is available regarding the remodeling of these TEVGs following implantation. Therefore, the primary goal of this study was to assess the feasibility and utility of a TEVG in the murine portal circulation in an effort to establish a preclinical animal model of TEPV implantation for noninvasive and mechanistic insight into the remodeling characteristics and translational potential of this regenerative medicine approach. In the present study, TEPVs were serially assessed *in vivo* from 1 to 12 months after implantation, at which point explanted neovessels resembled native PVs in terms of cellular composition and architecture, extracellular matrix composition and endothelial characteristics.

All implanted animals survived to predetermined experimental endpoints (up to 12 months postimplantation), which offers preliminary proof-of-concept of our TEPV approach in a small animal model. Additionally, implanted animals did not significantly differ from control animals with regard to body weight or spleen weight (Figure 1), indicating that animals implanted with TEPVs did not develop portal hypertension over the 12-month time course, as prior clinical studies have identified splenomegaly as a surrogate of portal hypertension [22].

In vivo ultrasound imaging demonstrated TEPV patency at serial time points for up to 12 months following implantation, with TEPV luminal diameters that did not significantly differ from those of native PVs in control animals (Figure 2). Additionally, micro-CT angiography confirmed *in vivo* TEPV patency in a subset of implanted animals, with TEPV luminal volumes and morphology that resembled native PVs (Figure 3).

In vivo findings of TEPV patency were further supported by postmortem histological analysis that demonstrated the formation of neovessels that resembled the vascular characteristics of native PVs after 12 months, with no

Figure 3. Serial assessment of *in vivo* micro-computed angiography. (A) Representative serial *in vivo* micro-computed tomography angiography at 2, 6 and 12 months after implantation in a TEPV demonstrates similar morphology to that of a native PV in control animals (location of TEPVs denoted by yellow arrows and markers). (B) Representative volume rendered *in vivo* micro-computed tomography angiography demonstrates a patent TEPV at 12 months after implantation (TEPV denoted by yellow markers). (C) Quantitative analysis of *in vivo* luminal volumes in TEPVs demonstrated similar morphology to native PVs during the 12-month experimental timeline. IVC: Inferior vena cava; PV: Portal vein; TEPV: Tissue-engineered portal vein.

differences in the content of smooth muscle cells, collagen type III or elastin between TEPVs and native PVs 12 months after implantation (Figure 4). Although smooth muscle cell content was not elevated at 12 months, significant differences in smooth muscle cell content were observed at 6 months postimplantation (Figure 4B). This increase in cell content observed at 6 months that is likely related to ongoing remodeling processes as the original scaffold transforms from graft to neovessel, a multistep process that is characterized by an inflammatory cascade resulting in an influx of cells [19,23], cellularization of graft, degradation of scaffold, proliferation of endothelial and

Figure 4. Serial histological analysis of tissue-engineered portal veins. (A) H&E, EVG and Masson's trichrome staining of native PV tissue and TEPVs explanted at 1, 2, 6 and 12 months after implantation. **(B)** Smooth muscle cell content was significantly higher in TEPVs compared with native PVs at 6 months after implantation, but did not significantly differ at 12 months after implantation. Elastin content was significantly lower in TEPVs at 6 months when compared with native PV tissue, but did not significantly differ at 12 months. TEPVs demonstrated significantly higher levels of collagen type 1 at 6 and 12 months after implantation, without significant differences in collagen type 3 at 6 or 12 months after TEPV implantation.

EVG: Elastica van Gieson; H&E: Hematoxylin and eosin; L: Lumen; mos: Months; PV: Portal vein; res: Resolution; TEPV: Tissue-engineered portal vein.

smooth muscle cells from the surrounding native vessels [24] and ultimate formation of a TEVG. In grafts that remain patent for up to 12 months, smooth muscle cell content decreases; however, in instances where increased number of cells persists, a known complication can be graft stenosis [10,13].

The significance of higher collagen and lower elastin content in this particular application of a TEVG within the portal circulation remains unclear (Figure 4B). In the portal circulation, TEPVs with less elastin could potentially have a decreased ability to dilate in the presence of portal hypertension. Future investigations in this animal model will need to focus on the biomechanical evolution of these vessels to fully understand the remodeling process and

Figure 5. Endothelial staining of (A) native portal vein and (B) TEPV tissue at 12 months after implantation. Presence of organized VE-cadherin (endothelial cells), eNOS and nuclei (DAPI) illustrate the development of a functional endothelium in a representative TEPV at 12 months after implantation. DAPI: 4',6-diamidino-2-phenylindole; TEPV: Tissue-engineered portal vein.

functional consequences of less elastin and higher collagen content. The TEPVs in this particular study properly functioned *in vivo* for up to 12 months postimplantation, which suggests that observed differences in elastin and collagen content do not result in TEPV failure. Additionally, although TEPVs did not completely mimic the histological properties of native PVs, TEPVs do appear to undergo a remodeling process that leads to the early stages of neovessel formation over a 12-month time course. Evidence supporting this notion was apparent in a representative TEPV at 12 months after implantation, which demonstrated the presence of VE-cadherin, eNOS and nuclei, thus representing the transition from a tissue-engineered vascular scaffold to a neovessel with the characteristics of a functional endothelium (Figure 5).

Conclusion

This study demonstrates feasibility of TEVGs in the portal circulation of a small animal model. Future application of TEPVs in a large animal model is warranted to fully elucidate the safety, efficacy and growth capacity of TEPVs, which may offer translational potential as meso-Rex bypass grafts and thus improve the care of children with EHPVO.

Executive summary

Tissue-engineered portal vein grafts are feasible in the portal circulation of a small animal model

- 12 mice were successfully implanted and survived for 12 months following implantation of tissue-engineered portal vein (PV) grafts (TEVGs).
- Animals did not develop evidence of portal hypertension over the 12-month experimental time course, as indicated by no changes in splenic weight.
- The murine model may provide a preclinical tool for future evaluation of the mechanisms associated with tissue-engineered PV (TEPV) remodeling.

TEPVs demonstrated patency for 12 months after implantation

- Serial *in vivo* ultrasound imaging demonstrated TEPV patency, with no evidence of stenosis, aneurysm formation or thrombosis.
- *In vivo* CT angiography revealed luminal volumes that resembled those of native PVs.

TEPVs resembled native PV architecture

- Histological analysis demonstrated similar content of smooth muscle cells, elastin and collagen type III between TEPVs and native PVs 12 months after implantation.

TEPVs began to develop endothelial characteristics similar to those of native PVs

- 12 months after implantation, endothelial staining of TEPV tissue demonstrated the presence of VE-cadherin (endothelial cells), eNOS and nuclei (4',6-diamidino-2-phenylindole).

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

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